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Rationally designed tularemia vaccines

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Abstract

Tularemia, caused by the Gram-negative bacterium *Francisella tularensis*, can be contracted by the bite of an arthropod vector or by inhalation. This disease occurs relatively infrequently but can be severe and even life-threatening if untreated. Until recently, there were few laboratories studying this organism; however, concerns over its potential use as a biological weapon have led to renewed attention to *F. tularensis* research, particularly in the area of vaccine development. Advances in the ability to genetically manipulate *F. tularensis*, along with knowledge gained from the creation and refinement of attenuated bacterial vaccines for other diseases, continue to foster significant progress in the development of live-attenuated bacterial vaccines, as well as defined antigen and subunit vaccines.

Keywords

biological warfare; *Francisella tularensis*; tularemia

Tularemia is caused by the Gram-negative bacterium *Francisella tularensis*, a zoonotic organism that can infect hundreds of different animal species. Humans are an incidental host, and there is no evidence for human-to-human transmission. Tularemia typically presents as an influenza-like illness, but symptoms may vary depending on the route of infection and the extent of systemic involvement [1]. Transmission generally occurs by the bite of an arthropod vector such as a tick, biting fly or mosquito, and these vector-mediated infections often present with lymphadenopathy and an ulcer at the site of entry [2]. Infections can also occur as a result of contact with contaminated material through a break in the skin or by inhalation. Inhalational exposures result in pneumonia but frequently more general symptoms, such as fever and malaise, are present and make the initial diagnosis more difficult.

There are several subspecies of *Francisella*. *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*, also known as type A and type B, respectively, are the most prevalent and are responsible for the majority of infections [3]. *F. tularensis* subsp. *mediasiatica* has lower virulence, and has only been isolated in central Asia [4]. *Francisella novicida* is a closely related, but less virulent, species that is often used as a model for *F. tularensis* subsp. *tularensis* pathogenicity; it is not pathogenic for immunocompetent humans, but is still highly virulent in mice. Type A isolates are classified as select agents of biological warfare by the

US Department of Health and Human Services and the Department of Agriculture primarily owing to the low lethal dose (≤ 10 organisms) and the ability for aerosol release and inhalational transmission. Type A isolates, typically restricted to North America, have the ability to cause a severe infection that is potentially fatal if untreated, whereas type B isolates are found throughout the Northern Hemisphere and cause a milder disease that is rarely fatal. Although type A and type B variants demonstrate considerably different degrees of virulence, comparisons among representative strains of each subspecies reveal that they share, on average, greater than 99% sequence identity at the genomic level [3,5]. The genetic basis for differences in virulence between subspecies is not entirely clear; however, bioinformatic analyses suggest that the formation of pseudogenes was a key driving force towards heightened infectivity in the more virulent subspecies [6,7].

The use of *Bacillus anthracis* (anthrax) as an agent of biological terror in the USA in 2001 revived interest in other potential biological warfare agents, including *F. tularensis*. The idea that an antibiotic or vaccine-resistant version of *F. tularensis* could be used as a biological terror agent created a renewed interest in developing a safe and effective vaccine for tularemia. An attenuated derivative of a type B strain, designated the live vaccine strain (LVS), has been used elsewhere as a fairly effective human vaccine for over 50 years. However, a number of issues continue to prevent its licensing and acceptance as a vaccine in the USA (reviewed in [8]). Included in these issues are the uncharacterized mechanisms of attenuation, which open up the possibility for reversion and difficulties in standardizing the manufacturing process, as well as unacceptable symptomatic side effects. For example, in early human vaccine trials aerosol immunization with LVS only partially protected against aerosol challenge with a type A strain. Immunization with a higher dose improved the level of protection, but 90% of the subjects receiving the higher dose actually developed mild tularemia, presenting with signs of fever, chest pain, headache and malaise [9]. One of the main goals of new vaccine efforts, and the emphasis of this review, is to develop a well-tolerated vaccine that protects against aerosol challenge with type A strains. Summaries of the candidate vaccines described in this review are listed in Table 1.

Immunity

F. tularensis is a facultative intracellular bacterium that can invade a variety of cell types including macrophages, endothelial cells and hepatocytes [10,11]. Once inside the cell, the bacterium escapes the phagosome and replicates within the cytoplasm [12,13]. Many infections with intracellular bacteria require cell-mediated immunity as a major component of the protective immune response; cell-mediated and humoral responses to *Francisella* are present in patients who have recovered from tularemia infection [12,13]. Vaccine studies in mice support this requirement for cell-mediated immune responses in protective immunity against *Francisella* infection, and demonstrate how this response differs against type A and type B strains [14-16]. Mice depleted of either CD4⁺ or CD8⁺ T cells, when immunized with LVS, are able to clear infection upon a secondary LVS challenge. However, depleting both T-cell subsets prevents clearance of the secondary LVS infection. Survival following secondary challenge with a type A strain, however, requires both CD4⁺ and CD8⁺ cells in addition to IFN- γ [14,15]. The humoral responses against type A and type B strains differ significantly as well, with antibodies playing a more protective role in the response against type B infection. Passive transfer of immune serum can completely protect against an aerosol challenge with LVS and is dependent upon the expression of Fc γ R on phagocytes, IFN- γ and α IgA antibodies [16,17]. Passive transfer does not, however, protect against similar challenge with a type A strain, although antibodies appear to make some synergistic contribution to protective immunity [18].

Live-attenuated vaccines

In the 1950s, Lee Forshay developed whole-killed bacteria and acetone extract vaccines for tularemia. Neither of these vaccines promoted significant protection in laboratory workers or in controlled animal trials [19,20]. The other major early vaccine approach involved a live-attenuated version of a type B *Francisella* strain. As part of an exchange program, Russia donated *Francisella* vaccine strains to the USA, including the strain now designated LVS, which were generated and selected through repeated laboratory passage [6]. Despite sequencing the entire LVS genome, and comparing the genomic sequence against wild-type strains, it remains unclear exactly which genetic changes contribute to its attenuation [5]. The ability of LVS vaccination to protect against an aerosol challenge of Schu S4 was tested on human volunteers [9]. Vaccination studies comparing scarification versus aerosol delivery determined that aerosol delivery was more efficacious, but the dose required for a high level of protection resulted in symptomatic side effects. The presence of protection nevertheless suggested the possibility of further LVS attenuation such that a higher dose could be tolerated and still achieve protective immunity. Genetic screens for attenuated mutants [21-25] and rationally designed mutants [26-28] exemplify some of the more recent attempts to create a new, defined live-attenuated vaccine without any symptomatic side effects, but which still elicits an effective protective immune response.

LVS-based vaccine candidates

The most promising attenuated LVS strain developed thus far is a mutant in iron superoxide dismutase gene (*sodB*) [28], which encodes an enzyme that breaks down superoxide radicals formed during aerobic respiration or during the respiratory burst of phagocytic cells. The LVS *sodB* mutant exhibited hypersensitivity to oxidative stress, and demonstrated reduced virulence by intranasal challenge when compared with LVS [29]. Intranasal immunization with this mutant elicited a better protective immune response than LVS against intranasal challenge with the Schu S4 type A strain; 40–42% of mice immunized with the *sodB* mutant survived a 103 colony-forming unit (CFU) challenge with Schu S4 versus 0% of the LVS-immunized mice.

Several investigators attempted to further attenuate LVS by mutating various genes encoding enzymes involved in lipopolysaccharide (LPS) biosynthesis. Li *et al.* constructed a mutant in *wbtI*, a gene encoding the transamine/perosamine synthetase of LPS O-antigen (O-Ag) [30]. This mutant produced no O-Ag, and had highly attenuated disease progression in mice when administered by both intradermal and intraperitoneal routes. Intraperitoneal and intradermal immunization with the *wbtI* mutant protected mice against a low dose challenge of LVS by intraperitoneal injection, but did not protect against a higher dose challenge. In the end, protection elicited by LVS immunization remained superior to that provided by the *wbtI* mutant because the LVS-immunized mice survived the higher dose challenge. This led the authors to surmise that O-Ag was an important stimulatory component of full protective immunity against LVS. Immunization with purified *Francisella* LPS or O-Ag affords protection against a lethal LVS challenge, but each contributes little to no protection against challenge with a type A strain [31,32]. LPS from many other Gram-negative bacteria are proinflammatory and can produce endotoxic shock; however, *F. tularensis* LPS is nontoxic and, therefore, an attractive vaccine candidate [33]. Intradermal immunization with O-Ag–bovine serum albumin (O-Ag–BSA) conjugate completely protected against intradermal challenge with a virulent type B isolate, but only delayed the time to death following aerosol challenge with this same isolate [31]. O-Ag–BSA conjugate immunization did not protect against intradermal or aerosol challenges with a type A strain.

Sebastian *et al.* constructed a mutant in *wbtA*, which encodes a dehydratase, and whose disruption also resulted in the absence of O-Ag surface expression. This mutant showed a high level of attenuation, whereby mice survived intranasal, intradermal and intraperitoneal doses

of 10^7 CFU [34]. When given as an intranasal or intradermal vaccine this mutant conferred protection against a lethal dose of LVS or a fully virulent type B clinical isolate by the homologous route of vaccination, but could not protect against intradermal Schu S4 challenge [34]. This improvement in protection of the LVS *wbtA* mutant over LVS is in contrast to LVS *wbtI*, which was a less effective vaccine than LVS [30]. One variation that could explain the difference in efficacy between these two vaccine trials is the route of challenge. Li *et al.* challenged immunized mice by intraperitoneal injection [30], whereas Sebastian and colleagues used intradermal and intranasal routes for their wild-type challenges [34].

In an attempt to improve vaccine efficacy of the LVS *wbtA* strain, Sebastian *et al.* combined intranasal immunization with the *wbtA* strain with subcutaneous immunization with LVS O-Ag conjugated to tetanus toxoid (O-Ag-TT) emulsified in Freund's adjuvant [18]. They hypothesized that together these two components would induce both strong humoral and cell-mediated responses, enhancing the efficacy of the vaccine. This combination vaccine completely protected against intranasal challenge with approximately 10 CFU of a virulent type B strain, and conferred 80% protection against an intranasal challenge of approximately 100 CFU. Protection against challenge with Schu S4 also improved, with 40% of the mice surviving an aerosol challenge of approximately 10 CFU and 60% surviving after intradermal challenge with approximately 1000 CFU. This combination vaccine, with its improved protection against intranasal challenge with a type A strain, suggests a role for robust humoral responses in enhancing vaccine efficacy against a type A strain.

Another successful approach in creating attenuated vaccine candidates for a variety of pathogens involves targeting the purine biosynthetic pathway [33,35,36]. Pechous *et al.* constructed an LVS mutant with a deletion in *purMCD* [26]. The resulting mutant was defective in intracellular replication and highly attenuated in mice via the intraperitoneal route. Intranasal immunization with as few as 5000 CFU of the mutant completely protected against an intraperitoneal challenge of 5000 CFU of LVS, whereas the LD₅₀ of LVS in naive animals by intraperitoneal injection was approximately 50 organisms. The same investigators created a similar *purMCD* mutant in Schu S4 [27]. The *purMCD* Schu S4 mutant also showed a high degree of attenuation when administered by intradermal or intranasal routes. Intranasal immunization with Schu S4 *purMCD* protected against intradermal challenges with Schu S4 or a virulent type B isolate; however, the immunization afforded the same level of protection against a moderate (100 CFU) Schu S4 intranasal challenge as that observed with mice immunized with wild-type LVS or LVS *purMCD*. No strain protected against a higher intranasal challenge (2000 CFU) of Schu S4. Unfortunately, there appeared to be tissue damage in mouse lungs following immunization with the Schu S4 *purMCD* mutant, an observation that raised concerns about whether a type A strain can be adequately attenuated for vaccine purposes.

Francisella novicida vaccine candidates

Although *F. novicida* is not pathogenic for humans, it is still fully virulent in mice. Because *F. novicida* does not have uncharacterized attenuating mutations like LVS, any identifiable attenuating mutations may potentially be more directly applicable to type A strains. *F. novicida* shares greater than 95% nucleotide similarity with type A and type B subspecies, so the majority of genes are present in all subspecies [7]. However, intradermal immunization with a sublethal dose of *F. novicida* provides no protection against intradermal or aerosol challenges with type A or type B strains, so it is unlikely that an attenuated strain of *F. novicida* would be directly useful as a tularemia vaccine [37].

Targeted mutations and transposon library screenings of *F. novicida* point to several loci as potential targets for attenuating mutations [23,24,38,39]. Kanistanon *et al.* mutated genes encoding enzymes involved in carbohydrate modifications of the lipid A component of LPS

[39]. Two mutants, *flmF2* and *flmK*, showed attenuation in mice when administered via subcutaneous and aerosol routes, and aerosol immunization provided protection against lethal aerosol challenge (25 CFU) with wild-type *F. novicida*.

Quarry *et al.* targeted the purine biosynthesis pathway, producing *purF* and *purA* mutants in *F. novicida* [38]. Both mutations provided at least 10,000-times more attenuation by intraperitoneal injection than the wild-type strain, but only immunization with the *purF* mutant provided protection against secondary intraperitoneal challenge with wild-type *F. novicida*. Immunization with the *purF* mutant did not protect against an intraperitoneal challenge with Schu S4.

Pammit *et al.* constructed a deletion mutant in *iglC* [40]. *IglC* is a novel essential virulence factor of *F. tularensis*, without which the bacteria cannot escape the phagosome and survive intracellularly [41,42]. Intranasal immunization with this *F. novicida* *iglC* mutant protected against intranasal challenges using 100- and 1000-times the LD₅₀ dose of wild-type *F. novicida*, resulting in 83 and 50% survival, respectively [40]. The successful protection of this *iglC* mutant against intranasal challenge suggested that *iglC* might be an appropriate candidate for an attenuating mutation in a type A strain [40]. Twine *et al.* constructed the *iglC* Schu S4 deletion mutant, a process that required an additional step because Schu S4 possesses two copies of this gene [43]. Although the mutant was highly attenuated, intradermal immunization with this strain failed to provide protection against both intradermal challenge with 500 CFU and intranasal challenge with approximately 10 CFU of Schu S4.

Tempel *et al.* identified 16 mutant strains defective in intra-cellular growth in macrophages from an *F. novicida* transposon library screen [24]. Intraperitoneal immunization with each of five of these 16 mutants, FTT0742, *pdpB*, *fumA*, *carB* and *dsbB*, provided protection against a high dose ($>8 \times 10^5$ CFU) intraperitoneal challenge with wild-type *F. novicida*. Although this particular study did not evaluate protection against Schu S4 challenge, it seems fairly clear that despite the high degree of genome similarity between *F. novicida* and type A and type B strains, an attenuated mutant in this lesser virulent *F. novicida* subspecies will not be effective as a protective vaccine against challenge with more virulent strains.

Type A vaccine candidates

The protective efficacies of further attenuated LVS mutants, such as the *sodB* mutant and the *wbtA*-O-Ag-TT combination vaccine, are much improved over parental LVS against respiratory challenges with type A strains. However, another obvious approach to vaccination is the development of an attenuated type A strain. Although LVS is closely related to Schu S4, genome sequence comparisons of LVS against type A strains identified 35 genes with altered protein sequences in LVS; 15 of these are predicted to be nonfunctional or defective in LVS [5]. Some of these proteins may be key protective antigens, or enhance infectivity in a manner that would improve immunogenicity. However, as the Schu S4 *purMCD* mutant illustrated [27], it is more challenging to use a more virulent type A background like Schu S4 and achieve a safe level of attenuation while still eliciting a high degree of immune protection. Twine *et al.* identified a spontaneous mutant of a type A strain, designated FSC043, with an intradermal LD₅₀ of greater than 10^8 CFU [43]. Comparative proteomics with Schu S4 and LVS revealed that both LVS and FSC043 lacked a 58-kDa protein, present in the parent Schu S4 strain and encoded by locus FTT0918. The subsequent construction of a Schu S4 strain with a deletion mutation in FTT0918 (Δ FTT0918) resulted in a strain with an intradermal LD₅₀ of approximately 10^5 CFU. Intradermal immunization with a sublethal dose of Δ FTT0918 protected mice against intradermal challenge with 500 CFU of the highly virulent type A strain FSC033 [43]. Aerosol immunization, followed by challenge with 10 CFU by the same route, protected only two out of six mice, but yielded a significant increase in the median time to

death over controls. There were no survivors in mice similarly immunized with LVS and then challenged with the type A strain.

Our lab constructed a *dsbB* mutant in a Schu S4 background and found that, although attenuated, it still retained some virulence with a LD₅₀ of approximately 10⁶ CFU [44]. Intranasal immunization with a sublethal dose of this *dsbB* Schu S4 mutant did not, however, confer protection against intranasal challenge with Schu S4. To more effectively identify an attenuated type A vaccine candidate, we screened a transposon mutagenesis library of Schu S4 for strains defective in intracellular growth in the hepatic cell line HepG2 [21]. One mutant had a transposon insertion in locus FTT1103, predicted to encode a novel lipoprotein, and appeared to be avirulent; mice survived infections by sub-cutaneous, intravenous and intraperitoneal routes, and intranasal challenges of greater than 10⁹ bacteria revealed no signs of infection or disease [45]. Although this mutant grew similarly to wild-type in culture, it was defective in intracellular growth in both HepG2 cells and the macrophage-like cell line J774A.1 and exhibited dissemination defects *in vivo*. Vaccine trials revealed that 83% of mice intranasally immunized with this mutant did not succumb to intranasal challenge with approximately 60 CFU of Schu S4. The success of this attenuated strain strongly supports the possibility for an attenuated type A strain as a safe vaccine.

Protective antigens

The ineffectiveness of the Forshay whole-cell killed vaccine provided an early indication that subunit vaccines would be unlikely candidates for a tularemia vaccine. However, the increase in protection observed during concomitant administration of purified O-Ag with the live O-Ag mutant indicated that the addition of key antigens to a vaccine formulation could be valuable [18]. Baron *et al.* also demonstrated that IL-12, when included as an adjuvant to mucosal vaccination with inactivated LVS, protected 90–100% of mice from lethal intranasal challenges with LVS [17]. Mice genetically deficient in IgA were not protected, indicating this protection was dependent upon an IgA response. These results, and those of the trials that follow, have revitalized the possibility for a subunit or defined antigen vaccine for tularemia.

Purified outer membrane proteins

Huntley *et al.* compared the efficacy of purified native outer membrane proteins (OMPs), ethanol-inactivated LVS and purified LVS LPS [46]. The vaccination protocol administered intraperitoneal injections with antigen emulsified in Freund's adjuvant at 0, 3 and 5 weeks, followed by intranasal challenge at 8 weeks with approximately 40 CFU of Schu S4. A total of 50% of OMP-immunized mice survived this Schu S4 challenge; those that succumbed to infection had significant increases in the median time to death. A total of 40% of mice immunized with inactivated LVS survived, while only 10% of LPS-immunized mice survived. OMP and inactivated LVS immunization independently elicited Th1- and Th2-associated antibody responses, but specific IgA was detectable only in mice immunized with LPS. Notably, the levels of protection against intranasal (aerosol) challenge achieved in this study trumped those of the most promising attenuated LVS candidates to date. With the prospect of new adjuvants for human use on the horizon, tularemia subunit vaccines may indeed be a viable option.

Antigen delivery via heterologous bacterial vector

Over the years researchers have devoted a considerable amount of effort towards developing live-attenuated bacteria for vaccine purposes, as well as using these strains as vehicles for delivering heterologous antigens [47]. One of the earliest approved live-attenuated vaccines was an attenuated strain of *Salmonella typhi* selected by chemical mutagenesis [48]. Today, we can apply more advanced genetic techniques in a similar capacity to target bacterial genes

for deletion or to stably express heterologous genes in an already attenuated strain. Attenuated *Salmonella typhimurium* presenting the *Francisella* antigens Tul4, a lipoprotein, and FopA, an OMP, both generated immune responses in mice. Although *S. typhimurium* expressing FopA provided no significant protection against subsequent LVS challenge [49], *S. typhimurium* expressing Tul4 showed some moderate protective ability demonstrated by decreased bacterial LVS load in immunized mice [50].

Jia *et al.* manipulated an attenuated strain of *Listeria monocytogenes*, another intracellular pathogen, by heterologously expressing *F. tularensis* proteins [51]. Attenuated strains of *L. monocytogenes* expressing recombinant proteins have previously promoted strong cell-mediated immune responses to the recombinant antigen upon immunization, some with remarkable efficiency [52]. For example, immunization with *L. monocytogenes* expressing a breast cancer tumor antigen significantly reduced metastatic disease in a mouse model [53]. *L. monocytogenes*, similar to *Francisella*, escapes from the phagosome into the cytoplasm; in this case, listeriolysin O promotes escape into the cytoplasm, and the bacterium can then propel itself from cell to cell by assembling actin filaments at one end using the actin nucleating protein, ActA [54]. These authors used an *actA* mutant of *L. monocytogenes*, previously demonstrated to be safely administered in humans, as the parent strain for *Francisella* protein expression [52]. They took several selected *F. tularensis* genes and cloned them, in-frame, after the sequence encoding the peptide signal sequence for listeriolysin O to target the proteins for secretion; to ensure stability, these recombinant genes were integrated into the bacterial chromosome. Of the six proteins tested, the expressed antigen yielding the most promising results was the essential virulence factor IglC. The IglC-expressing *Listeria* elicited no IglC-specific antibody, but did induce antigen-specific lymphocyte proliferation and IFN- γ production. Researchers intradermally vaccinated mice with live IglC-expressing *Listeria* and subsequently challenged by aerosolization with estimated doses of one lethal dose (1 $\times$) or ten-times a lethal dose (10 $\times$) of Schu S4, over a 15-min time period. When mice received two intradermal vaccinations with IglC-*Listeria*, 100% of mice survived the 1 $\times$ challenge, and 75% survived the 10 $\times$ challenge. However, variability in control mice appears to somewhat discount the remarkable efficacy observed in this trial; 50% of control-immunized mice survived the 1 $\times$ challenge and 87.5% of LVS immunized mice survived the 10 $\times$ challenge [52]. This survival data is inconsistent with previous LVS immunization studies which demonstrated that intradermal immunization with LVS did not protect against aerosol challenge with a type A strain, and that aerosol immunization with LVS was required to obtain at least some degree of protection against a similar aerosol challenge [14,55]. In this case, the survival of the LVS-immunized mice may reflect a difficulty in consistent aerosol delivery of the bacteria. Nonetheless, it remains impressive that a single antigen could induce a significant protective immune response. It was previously noted that immunization with an Schu S4 *iglC* mutant failed to provide protection against aerosol or intradermal challenges with a virulent type A strain [43]. Speculation that the failure of the *iglC* mutant to induce a protective immune response was due to its inability to escape the phagosome is not unfounded; retention in the phagosome could limit antigen presentation. Although phagosomal retention is likely the main preventative factor, the ability of IglC produced by *Listeria* to elicit a significant protective immune response suggests that the SchuS4 *iglC* mutant was also missing an important protective antigen.

Expert commentary

Tularemia is an infrequently occurring disease and generally responds well to treatment. Interest in a new vaccine stems mostly from its classification as a potential agent of biological warfare, and as such vaccine development to protect against the agent *F. tularensis* subsp. *tularensis* requires somewhat different criteria. The major threat of this organism lies in its ability to be disseminated by aerosolization, so vaccine efforts should be directed towards

protecting individuals from inhalational exposures with type A strains. Military personnel and first responders to biohazardous releases would be the most obvious beneficiaries of a tularemia vaccine. Mass vaccinations of a population would be unlikely, and perhaps only considered after a large release had occurred. In addition, since there is no human-to-human transmission, vaccination would only be advisable if repeat events were likely. Because the risk of exposure to *F. tularensis* is low, and somewhat unpredictable, a safe vaccine with as few side effects as possible should be a major criterion. However, in the event of a biological terror event this criterion could change if accelerated development becomes critical against engineered strains, particularly to protect against new multidrug-resistance strains.

Experience with LVS has demonstrated that an attenuated *Francisella* strain can be a safe vaccine with few side effects, at least when administered by a scarification route. The uncertainty with respect to the nature and impact of the attenuating mutations of LVS suggests that it would be advisable to start afresh from a wild-type strain. Making specific attenuating mutations in an unaltered type A parent strain, such as the wild-type Schu S4, should eliminate issues relating to the variable and reduced protection afforded from vaccination with an attenuated mutant derived from a different subspecies. We have shown that it is possible to safely attenuate a type A strain. However, because type A strains are the most virulent, care must be taken to ensure that any manipulated strain is sufficiently attenuated and that there is no possibility for reversion. This would perhaps be most easily assured by creating a strain with at least two attenuating and unlinked mutations where, ideally, each mutation alone provides the same level of attenuation and protective capability. However, the challenge in producing a new safe, defined live-attenuated vaccine, containing at least two attenuating mutations, will be to achieve a safe attenuation level and yet still retain the ability to mount an effective immune response.

Regardless, a live vaccine may encounter more difficulty in meeting regulatory approvals and licensing, particularly when the risk of exposure to the causative agent is low. Subunit vaccines are more attractive for safety reasons, particularly for immunocompromised patients. Immunizations with acellular and specific antigen components, such as O-Ag and OMPs, have been shown to possess an intrinsic efficacy and have a synergistic effect when administered in conjunction with a live-attenuated vaccine. The initial success of recombinant *L. monocytogenes* expressing *Francisella igIC* is a tantalizing 'compromise', as *L. monocytogenes* provides a better vehicle for antigen presentation. The safety of attenuated *L. monocytogenes* as a vaccine vector has previously been demonstrated, however, it would still be necessary to establish that the expression of a recombinant gene does not alter its safety profile.

A high level of efficacy is, of course, the essential component of any vaccine. Testing the efficacy of a vaccine on human populations, especially using an aerosol challenge, is not a viable option. Fortunately, *F. tularensis* is a zoonotic infection so there is no need to adapt or adjust animal models or strains to produce a virulent infection. Vaccine trials will, therefore, rely on the two-animal rule for both safety and efficacy. However, different animals demonstrate different susceptibilities to infection. For example, it is well established that mice are more susceptible to infection by *F. tularensis* subspecies than humans. The translational use of the mouse model to identify a protective vaccine for human populations is thus uncertain; the strain may be overattenuated and not capable of eliciting protective immunity, and/or the correlates of immunity might be different between species. Testing promising early vaccine candidates in a nonhuman primate model would provide some validation of the mouse model as a vaccine screening mechanism, and perhaps expedite the development of a safe and efficacious human tularemia vaccine.

Five-year view

The development of a tularemia vaccine has seen significant progress regarding safety and efficacy in recent years. It is clear that an effective tularemia vaccine that can protect against respiratory exposure will be possible. However, the jury is still out on which type of vaccine will be the most effective and appropriate. Direct work with virulent type A intranasal or aerosol challenges is just beginning, and there is still much to learn regarding correlates of immune protection against type A respiratory challenges. One challenge in the years ahead will be to enhance the limits of protection provided by various formulations, perhaps by combining vaccines and mixed prime-boost strategies of immunization.

If the number of individuals requiring vaccination remains limited (e.g., military personnel and first responders), subunit vaccines might be a viable option for a tularemia vaccine. However, the potential demand for mass vaccination imposes the need for a less expensive live-attenuated vaccine that could be quickly scaled up in a short period of time. Furthermore, the ability to rapidly produce large amounts of vaccine quickly would also reduce the need for stockpiling in preparation for an emergency. A recombinant *L. monocytogenes*, or perhaps another bacterial vector that is able to elicit protection against tularemia would be particularly attractive; not only would it be more cost effective, it would also entertain the possibility of combining *Francisella* antigens with those from other biological warfare agents to reduce the number of vaccines administered to at-risk populations.

Key issues

- New tularemia vaccines must protect against inhalational challenges with the most virulent type A strains.
- New defined, live-attenuated vaccines, within live vaccine strain (LVS) and Schu S4 backgrounds, have demonstrated improved safety and protection against intranasal type A challenge when compared with LVS.
- Antisera and antibodies specific to O-antigen of lipopolysaccharide significantly protect against intranasal type B strain challenges but, while they enhance protection against intranasal challenges, they cannot prevent infection with type A strains.
- Outer membrane preparations, purified O-antigen and attenuated *Listeria monocytogenes* expressing a protective *Francisella* antigen demonstrate protective abilities, reviving the possibility of using defined subunit vaccines for tularemia.

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References

Papers of special note have been highlighted as:

- of interest

- 1•. Tarnvik A, Berglund L. Tularaemia. Eur. Respir. J 2003;21(2):361–373. [PubMed: 12608453]• of interestProvides a concise review of the clinical aspects of tularemia.
2. Eliasson H, Broman T, Forsman M, Back E. Tularemia: current epidemiology and disease management. Infect. Dis. Clin. North Am 2006;20:289–311. [PubMed: 16762740]
3. Johansson A, Farlow J, Larsson P, et al. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J. Bacteriol 2004;186(17):5808–5818. [PubMed: 15317786]
4. Broekhuijsen M, Larsson P, Johansson A, et al. Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. J. Clin. Microbiol 2003;41(7):2924–2931. [PubMed: 12843022]
- 5•. Rohmer L, Brittnacher M, Svensson K, et al. Potential source of *Francisella tularensis* live vaccine strain attenuation determined by genome comparison. Infect. Immun 2006;74(12):6895–6906. [PubMed: 17000723]• of interestProvides comprehensive genome comparisons of *Francisella tularensis* subspecies *novicida* U112, *holarctica* live vaccine strain (LVS) and *tularensis* Schu S4.
6. Petrosino JF, Xiang Q, Karpathy SE, et al. Chromosome rearrangement and diversification of *Francisella tularensis* revealed by the type B (OSU18) genome sequence. J. Bacteriol 2006;188(19):6977–6985. [PubMed: 16980500]
7. Rohmer L, Fong C, Abmayr S, et al. Comparison of *Francisella tularensis* genomes reveals evolutionary events associated with the emergence of human pathogenic strains. Genome Biol 2007;8(6):R102. [PubMed: 17550600]
8. Conlan JW, Oyston PC. Vaccines against *Francisella tularensis*. Ann. NY Acad. Sci 2007;1105:325–350. [PubMed: 17395730]
9. Hornick RB, Eigelsbach HT. Aerogenic immunization of man with live tularemia vaccine. Bacteriol. Rev 1966;30(3):532–538. [PubMed: 5917334]
10. Conlan JW, North RJ. Early pathogenesis of infection in the liver with the facultative intracellular bacteria *Listeria monocytogenes*, *Francisella tularensis*, and *Salmonella typhimurium* involves lysis of infected hepatocytes by leukocytes. Infect. Immun 1992;60(12):5164–5171. [PubMed: 1452350]
11. Forestal CA, Benach JL, Carbonara C, Italo JK, Lisinski TJ, Furie MB. *Francisella tularensis* selectively induces proinflammatory changes in endothelial cells. J. Immunol 2003;171(5):2563–2570. [PubMed: 12928407]
12. Golovliov I, Baranov V, Krocova Z, Kovarova H, Sjostedt A. An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. Infect. Immun 2003;71(10):5940–5950. [PubMed: 14500514]
13. Clemens DL, Lee BY, Horwitz MA. Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. Infect. Immun 2004;72(6):3204–3217. [PubMed: 15155622]
- 14•. Conlan JW, Shen H, Kuolee R, Zhao X, Chen W. Aerosol-, but not intradermal-immunization with the live vaccine strain of *Francisella tularensis* protects mice against subsequent aerosol challenge with a highly virulent type A strain of the pathogen by an $\alpha\beta$ T cell- and interferon γ -dependent mechanism. Vaccine 2005;23(19):2477–2485. [PubMed: 15752834]• of interestShows the importance of T cells in immunity to type A strains.
15. Elkins KL, Cowley SC, Bosio CM. Innate and adaptive immunity to *Francisella*. Ann. NY Acad. Sci 2007;1105:284–324. [PubMed: 17468235]
16. Kirimanjeswara GS, Golden JM, Bakshi CS, Metzger DW. Prophylactic and therapeutic use of antibodies for protection against respiratory infection with *Francisella tularensis*. J. Immunol 2007;179(1):532–539. [PubMed: 17579074]
17. Baron SD, Singh R, Metzger DW. Inactivated *Francisella tularensis* live vaccine strain protects against respiratory tularemia by intranasal vaccination in an immunoglobulin A-dependent fashion. Infect. Immun 2007;75(5):2152–2162. [PubMed: 17296747]
- 18•. Sebastian S, Pinkham JT, Lynch JG, et al. Cellular and humoral immunity are synergistic in protection against types A and B *Francisella tularensis*. Vaccine 2009;27(4):597–605. [PubMed: 19022323]• of interestUsed a combination vaccine to enhance protective immunity and demonstrate a role for humoral immunity in protection against type A strains.

19. Van Metre TJ, Kadull P. Laboratory-acquired tularemia in vaccinated individuals: a report of 62 cases. *Ann. Intern. Med* 1959;50:621–632. [PubMed: 13627717]
20. Eigelsbach HT, Downs CM. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. *J. Immunol* 1961;87:415–425. [PubMed: 13889609]
21. Qin A, Mann BJ. Identification of transposon insertion mutants of *Francisella tularensis tularensis* strain Schu S4 deficient in intracellular replication in the hepatic cell line HepG2. *BMC Microbiol* 2006;6:69. [PubMed: 16879747]
22. Su J, Yang J, Zhao D, Kawula TH, Banas JA, Zhang JR. Genome-wide identification of *Francisella tularensis* virulence determinants. *Infect. Immun* 2007;75(6):3089–3101. [PubMed: 17420240]
23. Weiss DS, Brotcke A, Henry T, Margolis JJ, Chan K, Monack DM. *In vivo* negative selection screen identifies genes required for *Francisella* virulence. *Proc. Natl Acad. Sci. USA* 2007;104(14):6037–6042. [PubMed: 17389372]
24. Tempel R, Lai XH, Crosa L, Kozlowski B, Heffron F. Attenuated *Francisella novicida* transposon mutants protect mice against wild-type challenge. *Infect. Immun* 2006;74(9):5095–5105. [PubMed: 16926401]
25. Maier TM, Casey MS, Becker RH, et al. Identification of *Francisella tularensis* Himar1-based transposon mutants defective for replication in macrophages. *Infect. Immun* 2007;75(11):5376–5389. [PubMed: 17682043]
26. Pechous R, Celli J, Penoske R, Hayes SF, Frank DW, Zahrt TC. Construction and characterization of an attenuated purine auxotroph in a *Francisella tularensis* live vaccine strain. *Infect. Immun* 2006;74(8):4452–4461. [PubMed: 16861631]
27. Pechous RD, McCarthy TR, Mohapatra NP, et al. A *Francisella tularensis* Schu S4 purine auxotroph is highly attenuated in mice but offers limited protection against homologous intranasal challenge. *PLoS ONE* 2008;3(6):e2487. [PubMed: 18575611]
28. Bakshi CS, Malik M, Mahawar M, et al. An improved vaccine for prevention of respiratory tularemia caused by *Francisella tularensis* SchuS4 strain. *Vaccine* 2008;26(41):5276–5288. [PubMed: 18692537]
29. Bakshi CS, Malik M, Regan K, et al. Superoxide dismutase B gene (*sodB*)-deficient mutants of *Francisella tularensis* demonstrate hypersensitivity to oxidative stress and attenuated virulence. *J. Bacteriol* 2006;188(17):6443–6448. [PubMed: 16923916]
30. Li J, Ryder C, Mandal M, Ahmed F, Azadi P, Snyder DS, et al. Attenuation and protective efficacy of an O-antigen-deficient mutant of *Francisella tularensis* LVS. *Microbiology* 2007;153(Pt 9):3141–3153. [PubMed: 17768257]
31. Conlan JW, Shen H, Webb A, Perry MB. Mice vaccinated with the O-antigen of *Francisella tularensis* LVS lipopolysaccharide conjugated to bovine serum albumin develop varying degrees of protective immunity against systemic or aerosol challenge with virulent type A and type B strains of the pathogen. *Vaccine* 2002;20(29–30):3465–3471. [PubMed: 12297391]
32. Fulop M, Mastroeni P, Green M, Titball RW. Role of antibody to lipopolysaccharide in protection against low- and high-virulence strains of *Francisella tularensis*. *Vaccine* 2001;19(31):4465–4472. [PubMed: 11483272]
33. Jackson M, Phalen SW, Lagranderie M, et al. Persistence and protective efficacy of a *Mycobacterium tuberculosis* auxotroph vaccine. *Infect. Immun* 1999;67(6):2867–2873. [PubMed: 10338493]
34. Sebastian S, Dillon ST, Lynch JG, et al. A defined O-antigen polysaccharide mutant of *Francisella tularensis* live vaccine strain has attenuated virulence while retaining its protective capacity. *Infect. Immun* 2007;75(5):2591–2602. [PubMed: 17296751]
35. Kotloff KL, Pasetti MF, Barry EM, et al. Deletion in the *Shigella* enterotoxin genes further attenuates *Shigella flexneri* 2a bearing guanine auxotrophy in a Phase I trial of CVD 1204 and CVD 1208. *J. Infect. Dis* 2004;190(10):1745–1754. [PubMed: 15499528]
36. Wang JY, Pasetti MF, Noriega FR, et al. Construction, genotypic and phenotypic characterization, and immunogenicity of attenuated DeltaguaBA *Salmonella enterica* serovar typhi strain CVD 915. *Infect. Immun* 2001;69(8):4734–4741. [PubMed: 11447145]

37. Shen H, Chen W, Conlan JW. Mice sublethally infected with *Francisella novicida* U112 develop only marginal protective immunity against systemic or aerosol challenge with virulent type A or B strains of *F. tularensis*. *Microb. Pathog* 2004;37(2):107–110. [PubMed: 15312850]
38. Quarry JE, Isherwood KE, Michell SL, Diaper H, Titball RW, Oyston PC. A *Francisella tularensis* subspecies *novicida* *purF* mutant, but not a *purA* mutant, induces protective immunity to tularemia in mice. *Vaccine* 2007;25(11):2011–2018. [PubMed: 17241711]
39. Kanistanon D, Hajjar AM, Pelletier MR, et al. A *Francisella* mutant in lipid A carbohydrate modification elicits protective immunity. *PLoS Pathog* 2008;4(2):e24. [PubMed: 18266468]
40. Pammit MA, Raulie EK, Lauriano CM, Klose KE, Arulanandam BP. Intranasal vaccination with a defined attenuated *Francisella novicida* strain induces gamma interferon-dependent antibody-mediated protection against tularemia. *Infect. Immun* 2006;74(4):2063–2071. [PubMed: 16552035]
41. Santic M, Molmeret M, Klose KE, Jones S, Kwaik YA. The *Francisella tularensis* pathogenicity island protein IgIC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. *Cell. Microbiol* 2005;7(7):969–979. [PubMed: 15953029]
42. Lai XH, Golovliov I, Sjostedt A. Expression of IgIC is necessary for intracellular growth and induction of apoptosis in murine macrophages by *Francisella tularensis*. *Microb. Pathog* 2004;37(5):225–230. [PubMed: 15519043]
43. Twine S, Bystrom M, Chen W, et al. A mutant of *Francisella tularensis* strain SCHU S4 lacking the ability to express a 58-kilodalton protein is attenuated for virulence and is an effective live vaccine. *Infect. Immun* 2005;73(12):8345–8352. [PubMed: 16299332]
44. Qin A, Scott DW, Mann BJ. *Francisella tularensis* Schu S4 disulfide bond formation protein B, (DsbB), but not an RND-type efflux pump, is required for virulence. *Infect. Immun* 2008;76(7):3086–3092. [PubMed: 18458069]
45. Qin A, Scott DW, Thompson JA, Mann BJ. Identification of an essential *Francisella tularensis* subsp. *tularensis* virulence factor. *Infect. Immun* 2009;77(1):152–161. [PubMed: 18981253]• of interestIdentifies an avirulent mutant of a type A strain that can act as a protective vaccine.
46. Huntley JF, Conley PG, Rasko DA, Hagman KE, Apicella MA, Norgard MV. Native outer membrane proteins protect mice against pulmonary challenge with virulent type A *Francisella tularensis*. *Infect. Immun* 2008;76(8):3664–3671. [PubMed: 18505805]• of interestShows significant protection against a type A strain with an acellular vaccine.
47. Roland KL, Tinge SA, Killeen KP, Kochi SK. Recent advances in the development of live, attenuated bacterial vectors. *Curr. Opin. Mol. Ther* 2005;7(1):62–72. [PubMed: 15732531]
48. Kopecko DJ, Sieber H, Ures JA, et al. Genetic stability of vaccine strain *Salmonella typhi* Ty21a over 25 years. *Int. J. Med. Microbiol* 2008;299(4):233–246. [PubMed: 19121604]
49. Fulop M, Manchee R, Titball R. Role of two outer membrane antigens in the induction of protective immunity against *Francisella tularensis* strains of different virulence. *FEMS Immunol. Med. Microbiol* 1996;13(3):245–247. [PubMed: 8861037]
50. Sjostedt A, Sandstrom G, Tarnvik A. Humoral and cell-mediated immunity in mice to a 17-kilodalton lipoprotein of *Francisella tularensis* expressed by *Salmonella typhimurium*. *Infect. Immun* 1992;60(7):2855–2862. [PubMed: 1612751]
51. Jia Q, Lee BY, Clemens DL, Bowen RA, Horwitz MA. Recombinant attenuated *Listeria monocytogenes* vaccine expressing *Francisella tularensis* IgIC induces protection in mice against aerosolized type A *F. tularensis*. *Vaccine* 2009;27(8):1216–1229. [PubMed: 19126421]• of interestDemonstrates the successful use of a heterologous bacterial vector, whereby immunization with a single Francisella antigen can elicit protection against respiratory challenge with type A strains.
52. Bruhn KW, Craft N, Miller JF. *Listeria* as a vaccine vector. *Microbes Infect* 2007;9(10):1226–1235. [PubMed: 17719258]
53. Kim SH, Castro F, Gonzalez D, Maciag PC, Paterson Y, Gravekamp C. Mage-b vaccine delivered by recombinant *Listeria monocytogenes* is highly effective against breast cancer metastases. *Br. J. Cancer* 2008;99(5):741–749. [PubMed: 18728665]
54. Angelakopoulos H, Loock K, Sisul DM, Jensen ER, Miller JF, Hohmann EL. Safety and shedding of an attenuated strain of *Listeria monocytogenes* with a deletion of *actA/plcB* in adult volunteers: a

dose escalation study of oral inoculation. *Infect. Immun* 2002;70(7):3592–3601. [PubMed: 12065500]

- 55•. Chen W, Shen H, Webb A, KuoLee R, Conlan JW. Tularemia in BALB/c and C57BL/6 mice vaccinated with *Francisella tularensis* LVS and challenged intradermally, or by aerosol with virulent isolates of the pathogen: protection varies depending on pathogen virulence, route of exposure, and host genetic background. *Vaccine* 2003;21(25–26):3690–3700. [PubMed: 12922099]• of interest Describes differences in susceptibility and protection in different mouse backgrounds, and differences in protection against LVS and virulent type A and B strains.

Table 1

Candidate tularemia vaccine strains or antigens.

Mutation or antigen	Strain background	Protection (survival) against intranasal or aerosol challenge with type A strain	Ref.
<i>sodB</i> (superoxide dismutase)	LVS	40–42% protection against 103 CFU of Schu S4	[28]
<i>purMCD</i>	Schu S4 (type A)	Some protection against 100 CFU challenge, but not significantly improved over LVS or LVS <i>purMCD</i> immunization	[27]
	LVS (type B)	Similar protection as Schu S4 <i>purMCD</i>	[26]
O-Ag-BSA	Purified from LVS	No protection	[31]
LPS	Purified from LVS	No protection, but increased median time to death	[32]
<i>wbtA</i> (hydratase of O-Ag locus)	LVS	No protection	[33]
<i>wbtA</i> + O-Ag-TT	LVS	40% protection against 10 CFU	[18]
<i>wbtI</i> (transamine/perosamine synthetase of O-Ag locus)	LVS	Not tested	[30]
<i>flmF2</i> , <i>flmK</i>	<i>Francisella novicida</i>	Not tested	[39]
<i>purF</i>	<i>F. novicida</i>	No protection	[38]
<i>dsbB</i> , <i>carB</i> , <i>fumA</i> , <i>pdpB</i> , FTT0742	<i>F. novicida</i>	Not tested	[24]
<i>iglC</i>	<i>F. novicida</i>	Not tested	[40]
IglC	<i>Listeria monocytogenes</i>	100% survived 1× aerosol lethal challenge, 75% survived 10× challenge	[51]
OMP	Purified from Schu S4	50% protection against 40 CFU, increase in median time to death	[46]
FTT1103	Schu S4	83% protection against 60 CFU	[45]
FTT0918 (58-kDa protein)	Schu S4	Two out of six mice protected against 10 CFU, increased median time to death	[43]

BSA: Bovine serum albumin; CFU: Colony-forming unit; LPS: Lipopolysaccharide; LVS: Live vaccine strain; O-Ag: O-antigen; OMP: Outer membrane protein; TT: Tetanus toxoid.